Effects of Overexpression of Human GLUT4 Gene on Maternal Diabetes and Fetal Growth in Spontaneous Gestational Diabetic C57BLKS/J *Lepr*$^{db/+}$ Mice

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During gestation, heterozygous C57BLKS/J-*Lepr*$^{db/+}$ mice develop spontaneous gestational diabetes mellitus (GDM), and the newborn fetuses are macrosomic compared with offspring from wild-type (+/+) mothers. To investigate the effects of the leptin receptor mutation on maternal metabolism and fetal growth during pregnancy, we studied +/+, *db*/+, and *db*/+ transgenic mice that overexpress the human GLUT4 gene two- to threefold (*db*/+TG6). During pregnancy, fasting plasma glucose and hepatic glucose production were twofold greater in *db*/+ than +/+ mice, despite similar insulin levels. In skeletal muscle, insulin-stimulated tyrosine phosphorylation was decreased in pregnant +/+ mice, and even more so in *db*/+ mice: insulin receptor β (IR-β), +/+ 34%, *db*/+ 57% decrease, $P < 0.05$; insulin receptor substrate 1 (IRS-1), +/+ 44%, *db*/+ 61% decrease, $P < 0.05$; and phosphoinositol (PI) 3-kinase (p85α), +/+ 33%, *db*/+ 65% decrease, $P < 0.05$. Overexpression of GLUT4 in *db*/+TG6 mice markedly improved glucose-stimulated insulin secretion, by 250%, and increased IRβ, IRS-1, and p85α phosphorylation twofold, despite no change in concentration of these proteins. Plasma leptin concentration increased 40-fold during pregnancy, from 2.2 ± 0.5 to 92 ± 11 ng/ml and 3.6 ± 0.1 to 178 ± 34 ng/ml in +/+ and *db*/+ mice, respectively ($P < 0.01$), but was increased to only 23 ± 3 ng/ml in pregnant *db*/+TG6 mice ($P < 0.001$). Maternal fat mass and energy intake were greater in *db*/+ mice, and fat mass was reduced by GLUT4 overexpression, independent of food intake. Fetal body weight was increased by 8.1 and 7.9% in *db*/+ and *db*/+TG6 mothers, respectively ($P < 0.05$), regardless of fetal genotype, whereas fetuses from *db*/+TG8 mothers (four- to fivefold overexpression) weighed significantly less compared with pups from +/+ or *db*/+ mothers ($P < 0.05$). These results suggest that the single mutant *db* allele effects susceptibility to GDM through abnormalities in insulin receptor signaling, defective insulin secretion, and greater nutrient availability. GLUT4 overexpression markedly improves insulin-signaling in GDM, resulting in increased insulin secretion and improved glycemic control. However, maternal hyperglycemia appears not to be the sole cause of fetal macrosomia. These data suggest that GDM is associated with defects in insulin receptor signaling in maternal skeletal muscle, and this may be an important factor provoking maternal and fetal perinatal complications. *Diabetes* 48:1061–1069, 1999

Gestational diabetes mellitus (GDM), defined as impaired glucose tolerance, affects 3–5% of all human pregnancies and involves an interaction between diabetic susceptibility genes and the diabetogenic effects of pregnancy. Although GDM is generally reversible after pregnancy, women who develop GDM have up to a 50% risk of subsequent development of type 2 diabetes when they are not pregnant (1–3). In addition, the newborn fetuses of mothers who develop GDM are macrosomic and have higher concentrations of glucose after glucose tolerance tests and an increased prevalence of diabetes as adults compared with offspring from fathers who are diabetic (3–7).

During normal pregnancy, insulin-mediated glucose disposal decreases 40–60% from early to late pregnancy in humans (8) and rodents (9). Women with GDM appear to have abnormalities in insulin secretion that may contribute to the development of GDM (1,10–12). Some investigators have shown more pronounced insulin resistance in GDM patients compared with women with normal glucose tolerance during pregnancy (12–14); the insulin resistance may contribute to hyperglycemia in addition to defects in insulin secretion. The cellular mechanisms responsible for insulin resistance in skeletal muscle during pregnancy are unknown, but are probably related to defects in the insulin receptor signal cascade. The levels of GLUT4 in skeletal muscle are unchanged in women during pregnancy or in women with GDM (15). The intracellular events that couple the stimulation of insulin receptors to the movement of glucose transporters to the muscle cell surface are partially understood. Insulin stimulates the receptor to undergo autophosphorylation, thereby enhancing the tyrosine kinase activity of the β subunit of the receptor toward other protein substrates (16–18). Phosphorylation of insulin receptor substrate 1 (IRS-1) (and IRS-2) on

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E.M.G. holds stock in Pfizer.

ECL, enhanced chemiluminescence; GDM, gestational diabetes mellitus; IR, insulin receptor; IRS, insulin receptor substrate; PCR, polymerase chain reaction; PI, phosphatidylinositol; SGA, small for gestational age; STZ, streptozotocin; TG, transgenic; TNF, tumor necrosis factor.

multiple tyrosine residues after insulin treatment has been shown to be important in coupling the insulin receptor to glucose uptake. For example, in mice with a gene knockout of IRS-1, there is growth retardation and a mild form of glucose intolerance, including a 50% reduction in insulin-stimulated glucose transport in skeletal muscle and adipose tissue (19,20). IRS-1 phosphorylation results in the binding and phosphorylation of the regulatory subunit (p85α) of phosphatidylinositol (PI) 3 kinase to IRS-1 (21). Binding of the p85α isoform to tyrosine-phosphorylated IRS-1 increases catalytic activity of the PI 3 kinase complex (22). Formation of this protein complex appears to be necessary, although not sufficient, for stimulating glucose transport in 3T3-L1 adipocytes (23,24).

Most obese animals with genetic type 2 diabetes syndromes cannot be used as models for diabetes in pregnancy because of the very low fertility of the homozygotes, which is apparently related to an impaired hypothalamic-pituitary system resulting in decreased gonadotropin release. There are fertile heterozygotes of genetically determined diabetic animals with type 2 diabetes–like syndromes. Only a few experimental protocols have been carried out with such animals. Homozygous C57BLKS/J-*db*/*db* mice (also designated as C57BLKS/J-*Lepr*$^{db/db}$) are frankly diabetic and infertile, first going through a period of hyperglycemia with hyperinsulinemia and then losing the β-cell secretory capacity (25,26). However, impaired glucose tolerance could be elicited in heterozygous C57BLKS/J-*Lepr*$^{db/+}$ mice during pregnancy compared with normal C57BLKS/J-*Lepr*$^{+/+}$ mice (27,28). During gestation, heterozygous *db*/+ mice have abnormal glucose tolerance, postprandial hyperglycemia, and elevated glycohemoglobin compared with pregnant homozygous control (+/+) siblings. The fetuses from the diabetic pregnancies are macrosomic, with increased body, lung, and placenta weight (28,29). The newborn pups also exhibit abnormal carbohydrate metabolism, with increased liver glycogen, decreased blood glucose, and increased plasma insulin levels. Thus, the *db*/+ mouse model of spontaneous GDM exhibits many of the maternal-fetal abnormalities commonly found in offspring of diabetic mothers whose hyperglycemia is inadequately controlled.

The recent discovery of leptin and its mutant *db* receptor gene as regulators of appetite and energy expenditure suggest that one or more components of energy balance, interacting with the hormones of pregnancy, trigger glucose intolerance in the C57BLKS/J-*db*/+ mouse. The goal of these studies is to compare the effects of the leptin receptor mutation on maternal metabolism and fetal growth during pregnancy to possibly understand how defects in the maternal leptin receptor gene contribute to GDM, leading to fetal macrosomia (overgrowth). Because many metabolic disorders such as diabetes have both genetic and epigenetic components, we also sought to determine how modifying maternal insulin resistance during pregnancy decreases hyperglycemia and the development of fetal macrosomia by studying *db*/+ mice that overexpress the human GLUT4 gene. Several lines of these *db* transgenic mice have been characterized previously with regard to GLUT4 overexpression (about two- to fivefold in adipose, muscle, and heart), subcellular localization, and glucose transport properties (30,31).

RESEARCH DESIGN AND METHODS

Animals. Male and female C57BLKS/J-*Lepr*$^{+/+}$ and C57BLKS/J-*Lepr*$^{db+}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) at 6 weeks of age. Mice were housed individually and given free access to commercial mouse chow and water. Transgenic (TG) GLUT4 mice carrying 11.8 kb (*Not*I fragment) of the hGLUT4 transporter genomic DNA were produced as described previously (30). Two lines of homozygous GLUT4 female TG mice (hGLUT4*db*/+ lines 6 and 8) were used in this study. TG6 and TG8 overexpressed the GLUT4 gene two- to three- and four- to fivefold, respectively. At 60–70 days of age, the mice were fasted overnight and blood was obtained from the orbital sinus for measurement of insulin, glucose, and leptin concentrations. Female mice were then placed together with *db*/+ males, and mating was confirmed by the presence of a copulatory plug the next morning, designated day 0 of gestation. All living newborn pups were weighed to the nearest 0.01 g within the first 12 h after delivery at day 19 of gestation. Maternal weight gain was obtained by subtracting weight of the pups from maternal weight at the time of delivery. Fat mass was taken as the sum of mesenteric, gonadal, and retroperitoneal fat pads weighed to the nearest 0.01 g. Subcutaneous fat mass was not measured in these studies. The presence of the hGLUT4 transgene was assessed in pups by polymerase chain reaction (PCR) of total genomic DNA, as described (32). The mutant *db* gene was determined in DNA from fetuses using a PCR-based assay as published previously (33).

Glucose tolerance test. Glucose tolerance tests were performed before pregnancy and on day 18 of pregnancy after an overnight fast. The mice were bled via the orbital sinus (50 µl whole blood) before administration of glucose (2 g/kg body wt dextrose in 200 µl) injected intraperitoneally in conscious mice. Blood (30 µl) was sampled from the tail at 30, 75, and 120 min after glucose injection. Plasma glucose was measured by colorimetric glucose oxidase assay (Sigma, St. Louis, MO). The remaining blood sample was allowed to clot on ice and centrifuged for 20 min at 13,000 rpm at 4°C, and the serum was frozen at –70°C until analysis. Insulin and leptin were measured before pregnancy and at term using commercial radioimmunoassay kits for mice (Linco, St. Charles, IL). Assays were conducted in duplicate, and the intra-assay coefficient of variation was <5% for both assays.

Hepatic glucose production. In separate experiments, overnight fasting hepatic glucose production was measured in pregnant +/+ and *db*/+ mice at day 18 of gestation. Mice were injected with 5 µCi D-[3-^{3}H]glucose (New England Nuclear, Boston, MA) in 100 µl saline via the tail vein. Blood samples (25 µl) for glucose and radioactivity determinations were obtained from the tip of the tail at 5, 15, 30, 45, and 60 min. Serum was obtained after centrifugation at 5,000*g* for 5 min. Serum glucose levels were determined using the glucose oxidase method (Sigma). For radioactivity determinations, 10 µl blood was deproteinized with 200 µl of 20% trichloroacetic acid. Samples were centrifuged at 5,000*g* for 5 min, and the supernatants were evaporated to dryness overnight at 65°C under a hood. The residue was reconstituted in 200 µl water, 5 ml scintillation cocktail was added, and the samples were counted in a beta scintillation counter. The rate of hepatic glucose production was calculated using non–steady-state equations (34).

Indirect calorimetry. Before pregnancy, resting ventilation was assessed in awake, unanesthetized +/+ and *db*/+ mice by whole-body plethysmography. Briefly, mice were placed in an individual cylindrical Plexiglas test chamber measuring 3.25 inches high by 4.5 inches in diameter with a chamber volume of 600 ml. A Gilmont rotameter was used to maintain a bias flow rate of 300 ml/min room air through the chamber. The top of the chamber contained an opening through which gas samples were directed to the O_2 and CO_2 analyzers. The fractional contents of O_2 and CO_2 were determined using an AMETEK SA/1 O_2 analyzer and a Beckman LB-2 CO_2 analyzer. After 30–45 min, the steady-state oxygen consumption (V_{O_2}) and carbon dioxide production (V_{CO_2}) were calculated by determining the fractional content of O_2 and CO_2 in room air and within the chamber, multiplied by the flow rate, and corrected to standard temperature and pressure.

Tyrosine phosphorylation and Western blotting of insulin receptor, IRS-1, and p85α. Insulin-stimulated tyrosine phosphorylation of insulin receptor (IR), IRS-1, and the p85α regulatory subunit of PI 3 kinase was analyzed in skeletal muscle of intact mice during pregnancy by a method described previously (35), with minor modifications. On day 18, mice were fasted overnight and the next morning anesthetized with ketamine (150 mg/kg) and acepromazine (5 mg/kg), and the abdominal cavities were opened and the portal vein exposed. A 200-mg biopsy of the gastrocnemius was obtained and frozen immediately on dry ice. A 500-µl bolus of insulin (10 U/kg body wt) was then injected into the portal vein, and within 5 min, a sample from the opposite gastrocnemius muscle was quickly excised and frozen immediately. The frozen samples were homogenized under denaturing conditions, and proteins (100 µg) from the muscle were resolved on 7% SDS gel, transferred to Immobilon membrane, and detected with polyclonal antibodies against IRβ, IRS-1, p85α, and horseradish peroxidase IgG using enhanced chemiluminescence (ECL) (35). For immunoprecipitation, 4 mg muscle protein was incubated overnight at 4°C with an anti-phosphotyrosine antibody (5 µg antibody/8 mg protein) in 1 ml immunoprecipitation buffer containing 2% Triton-X-100, 300 mmol/l NaCl, 20 mmol/l Tris-HCl, 2 mmol/l EDTA, 2 mmol/l EGTA, 0.4 mmol/l phenylmethylsulfonyl fluoride (PMSF), 0.4 mmol/l sodium vanadate, and 1% NP-40. After immunoprecipitation, the samples were mixed with 50 µl protein-A sepharose (10% solution) for 4 h at 4°C, and the immunoprecipitate was washed in 1 ml immunoprecipitation buffer, followed by centrifugation at 500*g* for 1 min at 4°C, repeated four times. The washed precipitate was mixed with Laemmli sample buffer (50 µl), boiled for 5 min, and centrifuged for 5 min at 500*g*, and the super-

natant (20 µl) was separated using 7% Tris polyacrylamide gel electrophoresis on a Bio-Rad Mini-Protein gel apparatus. Proteins were then electrotransferred from the gel to polyvinylidine difluoride (PVDF) membrane at 100 V (constant current) for 2 h using a minitransfer apparatus (Idea Scientific, Minneapolis, MN). Gels were stained with Coomassie blue to ensure equal protein transfer. Autoradiography was carried out using Kodak XAR X-ray film. After treatment with the ECL reagent, the exposure time was varied from 1 to 3 min, and each exposure was quantified by densitometry. The specific band intensities were quantitated by optical densitometry using a Digiscan scanner (U.S. Biochemical, Cleveland, OH). Each muscle sample was run an average of three separate assays using different minigels.

Statistical analysis. Results are presented as means ± SE for the indicated number of mice. Comparisons between groups were made using Student's unpaired *t* test, except for glucose tolerance data, which was analyzed by analysis of variance for repeated measures using Prism (Graph Pad Software, San Diego, CA). Statistical significance was set at $P < 0.05$.

RESULTS

Changes in energy intake, body weight, and fat mass during pregnancy. Before pregnancy, there were no differences in body weight or food intake measured in +/+ compared with *db*/+ mice (Table 1). However, fat mass was 31 ± 17% greater ($P < 0.01$) in age-matched nonpregnant *db*/+ mice. During pregnancy, the average food intake was significantly greater by 19 ± 5% in both *db*/+ and *db*/+TG6 mothers ($P < 0.01$) compared with pregnant +/+ mice. Increased energy intake in pregnant *db*/+ mice coincided with a significant 34 ± 7% greater maternal weight gain and a 34 ± 15% greater fat mass ($P < 0.01$) compared with pregnant +/+ mice. In *db*/+TG6 mice, weight gain and fat mass were significantly less than in *db*/+ mice ($P < 0.01$), despite similar energy intake. Energy expenditure was measured by indirect calorimetry before pregnancy and was significantly lower by 12% in *db*/+ compared with +/+ mice (10.41 ± 0.01 vs. 11.55 ± 0.01 ml · kg^{-1} · min^{-1}, $P < 0.05$, data not shown).

Development of pregnancy-induced glucose intolerance. As shown in Fig. 1, pregnant *db*/+ mice demonstrated a profound glucose intolerance during the glucose challenge test, with significantly higher glucose compared with pregnant +/+ mice ($P < 0.01$). In marked contrast, pregnant *db*/+TG6 mice had glucose levels similar to +/+ mice throughout the glucose tolerance test. Based on glucose tolerance tests during pregnancy, there was virtually 100% penetrance in the *db*/+ mice (30 min blood glucose >250 mg%). Likewise, the effect of the GLUT4 transgene restored normal glucose tolerance in all of the animals examined. Fasting insulin levels in pregnant +/+ and *db*/+ mice were not significantly different. During the glucose challenge, pregnant +/+ mice showed a doubling of circulating insulin levels at 30 min, while *db*/+ pregnant mice increased by 50% from 0 to 30 min. In *db*/+TG6 mice, there was a 327% increase in insulin levels during the glucose challenge test, with significantly higher insulin levels at 30 and 75 min compared with *db*/+ mice ($P < 0.01$).

The plasma glucose, insulin and leptin levels for all the mice studied are summarized in Table 2. During pregnancy, *db*/+ mice had a twofold greater plasma glucose response to a glucose load. Insulin levels were similar in +/+ and *db*/+ mice. In contrast, *db*/+TG6 mice had a normal glucose response with a twofold greater insulin secretion compared with *db*/+ mice ($P < 0.01$). The *db*/+TG8 mice displayed normal glucose levels with approximately threefold lower insulin compared with *db*/+TG6 mice ($P < 0.01$). The lower insulin levels in *db*/+TG8 mice correlates well with an approximate fourfold increase in GLUT4 transporter protein at the cell surface of skeletal muscle in the basal and insulin-stimulated state compared with wild-type mice (30,31).

Plasma leptin concentration increased markedly during pregnancy in +/+ mice, from 2.2 ± 0.5 to 92 ± 11 ng/ml, and in *db*/+ mice, from 3.6 ± 0.1 to 178 ± 34 ng/ml ($P < 0.01$). The leptin results in TG8 mice were similar to TG6 and averaged together. The leptin levels in TG6 mice were significantly decreased to 23 ± 3 ng/ml ($P < 0.001$). Pregnant *db*/+TG8 mice were similar to TG6 Mice. Hepatic glucose production was measured in five *db*/+ and four +/+ mice during day 18 of gestation, and was doubled in *db*/+ compared with +/+ mice (41.2 ± 8.5 vs. 20.7 ± 5.31 mg · kg^{-1} · min^{-1}, $P < 0.05$, data not shown).

Effect of pregnancy and GDM on skeletal muscle insulin receptor, IRS-1, and p85α phosphorylation and protein concentrations. To determine the biochemical mechanism underlying impaired glucose tolerance in the pregnant *db*/+ mouse, several aspects of the insulin receptor signal transduction network were assayed in skeletal muscle of pregnant +/+, *db*/+, and *db*/+TG6 mice. Control and pregnant mice (day 19) were injected with insulin, and 5 min later the gastrocnemius muscle was removed and homogenized as described in METHODS. An example autoradiogram is shown in Fig. 2*A*, and the results of multiple experiments were quantified by scanning densitometry. Insulin increased insulin receptor β-subunit tyrosine phosphorylation up to 10-fold in muscles of nonpregnant +/+ mice. During pregnancy, however, insulin-stimulated receptor autophosphorylation decreased by 34 ± 7 and 57 ± 6% ($P < 0.01$) in pregnant +/+ and *db*/+ mice, respectively, compared with nonpregnant +/+ mice. The patterns for IRS-1 and p85α subunit tyrosine phosphorylation were similar, with 35–45 and 61–65% reductions in +/+ and *db*/+ mice, respectively ($P < 0.01$). Furthermore, the levels of IRβ, IRS-1, and p85α

TABLE 1
General characteristics of nonpregnant and pregnant C57BLKS/J-*Lepr*$^{+/+}$, C57BLKS/J-*Lepr*$^{db/+}$, and *db*/+TG6 mice

	Body weight (g)	Food intake (g/d)	Weight gain (g)	Fat mass (g)
Nonpregnant				
+/+	22.6 ± 0.8	3.3 ± 0.1	—	0.45 ± 0.05
db/+	23.8 ± 0.8	3.4 ± 0.2	—	0.59 ± 0.08*
Pregnant				
+/+	32.1 ± 1.4	4.3 ± 0.1	2.9 ± 0.12	0.71 ± 0.12
db/+	33.9 ± 1.3	5.1 ± 0.4*	3.9 ± 0.21*	0.95 ± 0.11*
db/+TG6	31.3 ± 2.3	5.0 ± 0.2	2.1 ± 0.10†	0.51 ± 0.13†

Data are means ± SE; 6–10 animals per group. Measurements in pregnant mice were made on day 19 of gestation. *$P < 0.01$ between *db*/+ and +/+ mice in the same condition; †$P < 0.01$ between pregnant *db*/+ and pregnant *db*/+ TG6 mice.

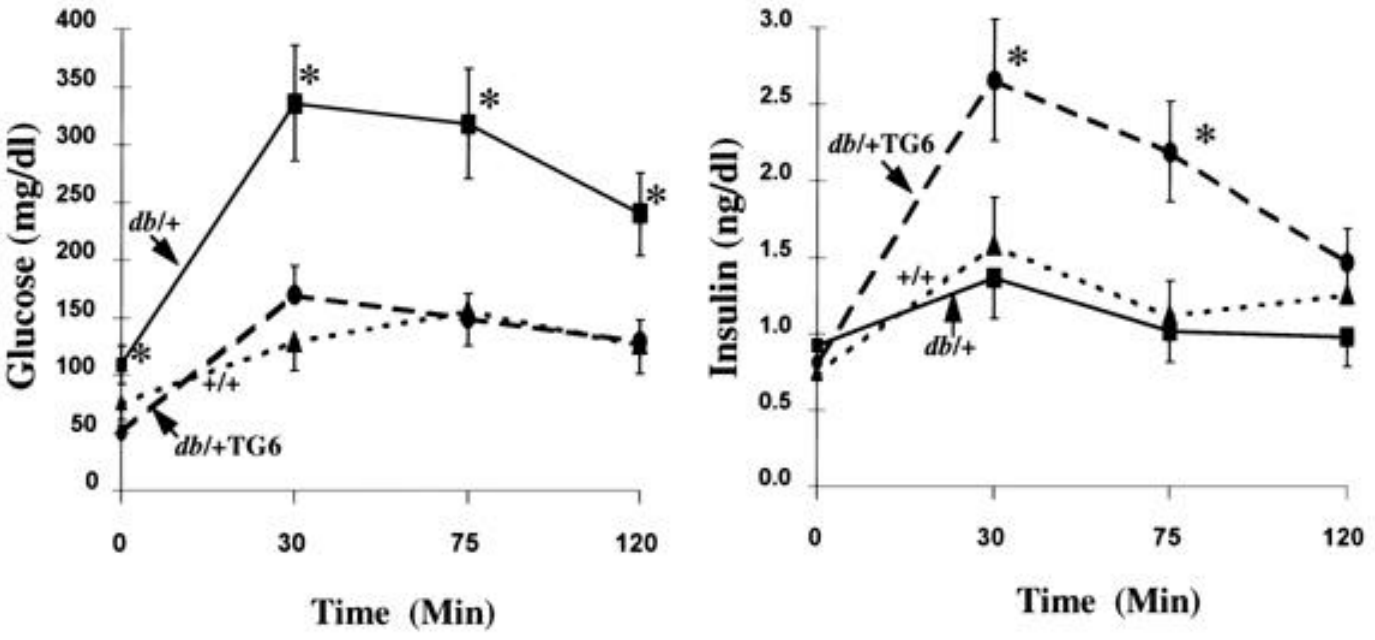

FIG. 1. Plasma glucose and serum insulin concentrations during an intraperitoneal glucose tolerance test in pregnant +/+, *db*/+, and *db*/+TG6 mice at day 18 of gestation. Mice were fasted overnight and administered 2 g/kg body wt glucose load at time 0, and glucose and insulin levels were determined at various time points. Values are means ± SE for six to eight mice per group. *$P < 0.01$.

phosphorylation were significantly lower in the muscles of the pregnant *db*/+ mice compared with pregnant +/+ mice ($P < 0.05$). As shown in Fig. 2*B*. there were no changes in the insulin receptor protein levels in muscle as shown by immunoblotting with an antibody to the COOH terminus of the insulin receptor. In contrast, the levels of IRS-1 protein were significantly reduced by 40% ($P < 0.01$) in muscles from both +/+ and *db*/+ mice compared with nonpregnant +/+ mice. The levels of p85α protein were not significantly different in muscle from pregnant mice.

Effect of overexpression of human GLUT4 gene on *db*/+ pregnant mice and fetal weight. Figure 3*A* shows a representative example comparing insulin-induced tyrosine phosphorylation in skeletal muscle from pregnant *db*/+ and *db*/+TG6 mice. In *db*/+TG6 mice, insulin increased the level of tyrosine phosphorylation of IRβ, IRS-1, and p85α by 210 ± 23, 185 ± 16, and 202 ± 21%, respectively ($P < 0.01$), over the levels in pregnant *db*/+ mice. Despite these increases in tyrosine phosphorylation, there were no changes observed in the levels of IRβ, IRS-1, or p85α signaling proteins in muscles of the *db*/+TG6 mice compared with pregnant *db*/+ mice (Fig. 3*B*).

Table 3 illustrates the effect of maternal diabetes and GLUT4 overexpression on fetal birth weight. Compared with pups born to +/+ mothers, the average fetal body weight was increased 8.1 ± 1.6 and 7.85 ± 1.6% from *db*/+ and *db*/+TG6 mothers, respectively ($P < 0.05$), regardless of fetal genotype. Fetuses from *db*/+TG8 mothers were significantly underweight by 11 ± 1.1 and 17.3 ± 0.9% compared with fetuses from +/+ or *db*/+ mothers, respectively ($P < 0.05$). The numbers of fetuses born to +/+, *db*/+, and *db*/+TG6 dams were not different, while the number of pups born to *db*/+TG8 mice was significantly reduced, by ~ 40% compared with +/+ mothers ($P < 0.01$).

TABLE 2
Plasma glucose, insulin, and leptin concentrations in nonpregnant and pregnant C57BLKS/J-+/+, C57BLKS/J-*db*/+, and *db*/+GLUT4 mice

	Plasma glucose (mg/dl)	Serum insulin (ng/ml)	Serum leptin (ng/ml)
Nonpregnant			
+/+	157 ± 18	1.16 ± 0.11	2.2 ± 0.5
db/+	173 ± 28	1.04 ± 0.21	3.6 ± 0.1*
Pregnant			
+/+	159 ± 19	1.37 ± 0.22	98 ± 11
db/+	318 ± 16*	1.27 ± 0.27	178 ± 34*
db/+TG6	186 ± 23†	2.66 ± 0.38†	23 ± 3†
db/+TG8	146 ± 26	0.84 ± 0.20‡	—

Data are means + SE; 6–10 animals per group except *db*/+TG8 ($n = 4$). Measurements in pregnant mice were made on day 18 of gestation. Values for plasma glucose and serum insulin were obtained 30 min after an intraperitonal glucose challenge (2 g/kg body wt). TG8 and TG6 mice values were pooled for leptin measurements. *$P < 0.01$ between *db*/+ and +/+ mice in the same condition; †$P < 0.01$, between pregnant *db*/+ and pregnant *db*/+ TG6 mice; ‡$P < 0.01$ between *db*/TG8 and *db*/+TG6 mice.

DISCUSSION

A number of animal models have been used to investigate mechanisms underlying maternal diabetes and macrosomia in infants of diabetic mothers. Most of these models have been derived by either eliminating maternal insulin production by streptozotocin (STZ) injection or by giving large amounts of glucose or insulin to the mother or fetus. Fetuses of some of these animals, particularly the STZ-treated rat with profound maternal and fetal hyperglycemia, are actually small for gestational age rather than macrosomic (36). In addition, women diagnosed with GDM have impaired glucose tolerance, usually during the third trimester, associated with moderate to severe insulin resistance. Thus, the heterozygous *db*/+ mouse, in which a specific genetic lesion leads to

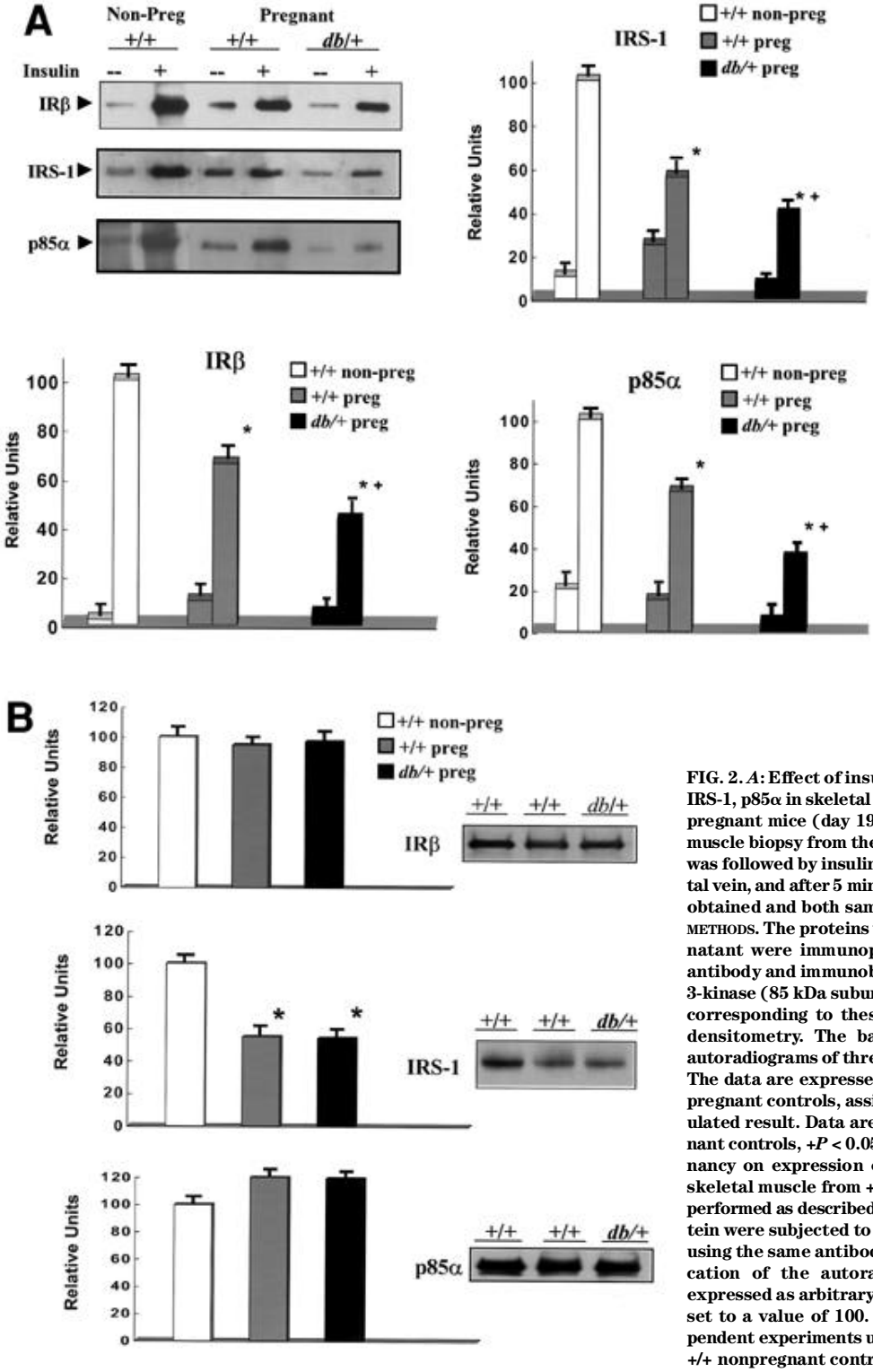

FIG. 2. ***A*: Effect of insulin on tyrosine phosphorylation of IRβ, IRS-1, p85α in skeletal muscle of +/+ and *db*/+ mice. Control and pregnant mice (day 19) were anesthetized before obtaining a muscle biopsy from the gastrocnemius muscle (Insulin –). This was followed by insulin injection (10 U/kg body wt) via the portal vein, and after 5 min, a second muscle sample (Insulin +) was obtained and both samples were homogenized as described in METHODS. The proteins were isolated, and aliquots of the supernatant were immunoprecipitated with antiphosphotyrosine antibody and immunoblotted with anti IRβ, IRS-1, and anti-PI 3-kinase (85 kDa subunit). The tyrosine-phosphorylated bands corresponding to these proteins were analyzed by scanning densitometry. The bar graphs show quantification of the autoradiograms of three experiments using six mice per group. The data are expressed as arbitrary units relative to +/+ nonpregnant controls, assigning a value of 100 to the insulin-stimulated result. Data are means ± SE. *$P < 0.01$ vs. +/+ nonpregnant controls, +$P < 0.05$ vs. +/+ pregnant mice. *B*: Effect of pregnancy on expression of insulin receptor, IRS-1, and p85α in skeletal muscle from +/+ and *db*/+ mice. Tissue extraction was performed as described in *A* and METHODS. Equal amounts of protein were subjected to SDS-PAGE and Western immunoblotted using the same antibodies. The bar graphs show data quantification of the autoradiograms by scanning densitometry, expressed as arbitrary units relative to +/+ nonpregnant mice, set to a value of 100. Data are means ± SE from three independent experiments using six mice in each group. *$P < 0.01$ vs. +/+ nonpregnant controls.**

defects in both insulin secretion and insulin action during pregnancy, would appear to be an ideal model to study the interaction between diabetic susceptibility genes and maternal-fetal metabolism.

Before pregnancy, we found decreased energy expenditure and greater fat mass in *db*/+ mice compared with +/+ mice, suggesting that a single copy of the mutant *db* gene may be permissive for the development of obesity. We did not measure energy expenditure in pregnancy, but we found that pregnant *db*/+ mice had greater energy intake and maternal weight gain compared with +/+ mice, suggesting that increased caloric intake and possibly decreased energy expenditure may increase adiposity and diabetic susceptibility in pregnant *db*/+ heterozygotes. We also found that the *db*/+ mice had inadequate insulin secretion to maintain normoglycemia during pregnancy. There is good evidence that hyperglycemia increases the severity of defects in both β-cell function and skeletal muscle insulin resistance (37). The cellular mechanisms underlying reduced insulin signal transduction in pregnancy are unknown but may be related to the hormonal milieu of pregnancy. An increase in maternal plasma corticosterone has been observed during early gestation, and there is evidence that treatment with excess glucocorticoids can reduce tyrosine phosphorylation of IRβ and IRS-1 as well as PI-3 kinase activity in skeletal muscle and liver (38). Interestingly, we also observed a reduction in the expression of IRS-1 in human pregnancy (J.E.F., P. Catalano, unpublished observations). These results are similar to those of Saad et al. (39), who reported that insulin receptor autophosphorylation was reduced by 30% in muscle and liver of pregnant rats at day 20

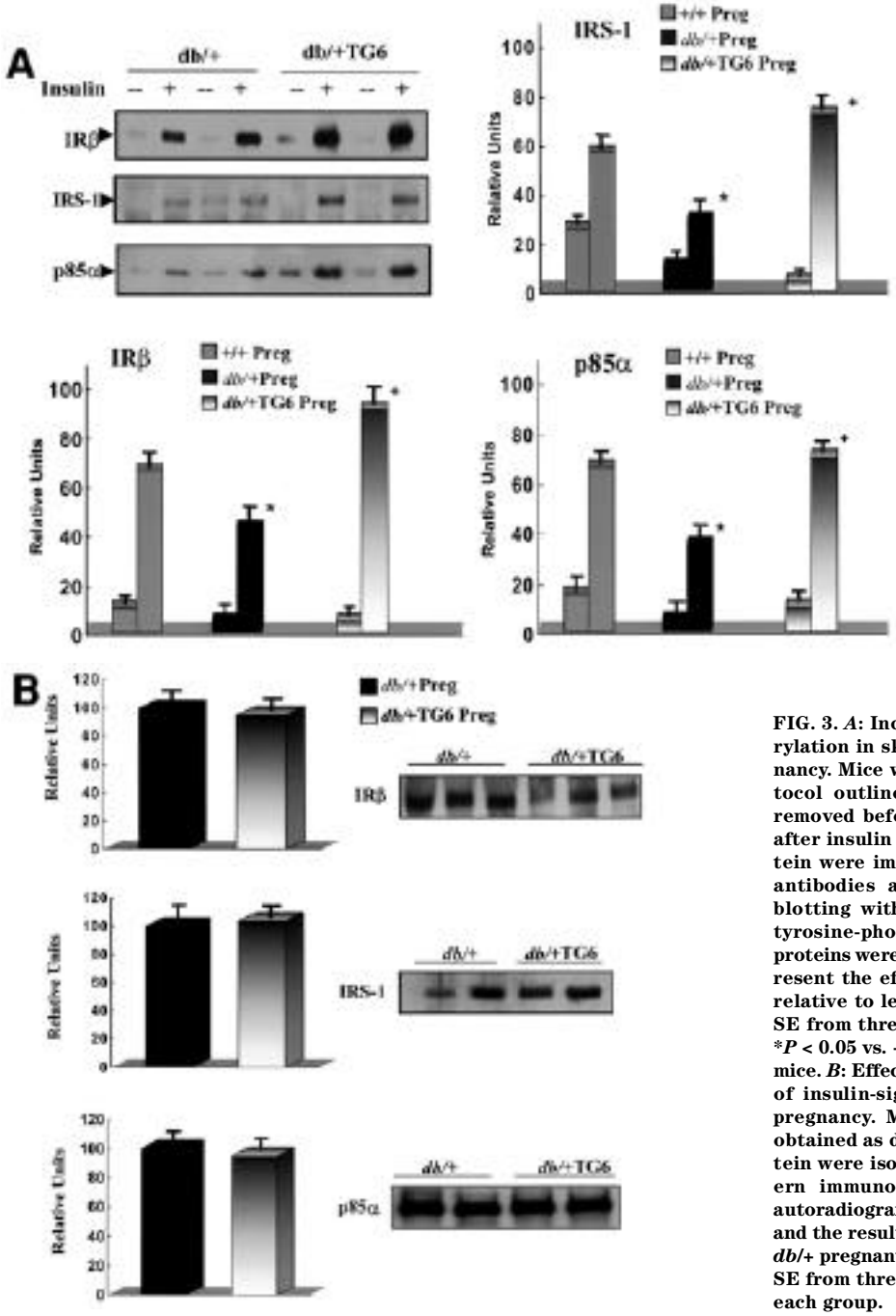

FIG. 3. ***A*: Increased insulin-stimulated tyrosine phosphorylation in skeletal muscle of *db*/+TG6 mice during pregnancy. Mice were anesthetized and treated using the protocol outlined in Fig. 2*A* and METHODS. Muscles were removed before insulin treatment (Insulin –) and 5 min after insulin injection (Insulin +). Equal amounts of protein were immunoprecipitated with antiphosphotyrosine antibodies and subjected to SDS-PAGE and Western blotting with antibodies to IRβ, IRS-1, and p85α. The tyrosine-phosphorylated bands corresponding to these proteins were scanned by densitometry. The bar graphs represent the effect of insulin on tyrosine phosphorylation, relative to levels in +/+ pregnant mice. Data are means ± SE from three experiments using six mice in each group. ****P* < 0.05 vs. +/+ pregnant mice; +*P* < 0.01 vs. *db*/+ pregnant mice. *B*: Effect of GLUT4 overexpression on the expression of insulin-signaling proteins in skeletal muscle during pregnancy. Muscles from *db*/+ and *db*/+TG6 mice were obtained as described in *A*. Equal amounts of muscle protein were isolated and subjected to SDS-PAGE and Western immunoblotting using the same antibodies. The autoradiograms were quantified by scanning densitometry, and the results are expressed as arbitrary units relative to *db*/+ pregnant mice, set to a value of 100. Data are means ± SE from three independent experiments using six mice in each group.**

TABLE 3
Average fetal birth weight and litter size born to wild-type (+/+), heterozygous *db*/+, and *db*/+ hGLUT4 mice

	+/+	*db*/+	*db*/+TG6	*db*/+TG8
Birth weight (g)	1.23 ± 0.02	1.33 ± 0.02*	1.31 ± 0.03*	1.10 ± 0.02*
n	56	66	41	13
Litter size (*n*)	5.5 ± 0.4	6.1 ± 0.3	5.8 ± 0.3	3.6 ± 0.2*

Data are means ± SE. *$P < 0.01$, significantly different from +/+ mice.

of gestation, while IRS-1 protein levels decreased by 45 to 56% in liver and muscle of pregnant rats. These findings suggest that changes in the early steps of insulin-signaling cascade may play a role in insulin resistance in pregnancy, and that reduced tyrosine phosphorylation of the insulin receptor and related proteins may provoke further muscle insulin resistance in GDM. Although the transgenic mice and single gene mutations used here cannot substitute for studying GDM in patients, these animals have many of the obvious symptoms of GDM, including insulin-signaling defects in insulin-sensitive tissues and defects in insulin secretion.

Our results using *db*/+TG6 mice show that GLUT4 overexpression can increase IRβ, IRS-1, and p85α phosphorylation by twofold in skeletal muscle, despite no change in expression of these proteins. How tyrosine phosphorylation is modified by increased GLUT4 is unknown, but it is possible that improvement is acquired as a result of the correction in glucose homeostasis. In humans with type 2 diabetes and GDM, there is good evidence that insulin-signaling defects exist in muscles and adipose tissue, as well as GLUT4 translocation defects. Whether increased GLUT4 is associated with improved insulin-signaling in patients with GDM is not known. However, increased insulin-signaling was associated with improved glucose disposal during an OGT, and thus this adaptation, in addition to increased GLUT4, may be part of the mechanism for improved glucose tolerance. The involvement of metabolic fuels such as free fatty acids, ketone bodies, and diacylglycerol in pregnancy-related modifications of insulin receptor signaling has received little attention. The cytokine tumor necrosis factor (TNF)-α has recently been proposed to play a role in the insulin resistance of obesity and type 2 diabetes (40). Infusion of TNF-α causes insulin resistance in rats and in human skeletal muscle cells incubated in culture by interfering with IR and IRS-1 phosphorylation (40,41). TNF-α mRNA expression is elevated in the adipose tissue of obese animals and in humans with obesity (42), and the circulating levels correlate well with the extent of insulin resistance measured in humans during pregnancy (43). The fat content in pregnant *db*/+ mice was increased compared with +/+ mice and was significantly reduced in *db*/+TG6 mice, suggesting perhaps that circulating TNF-α or possibly free fatty acids could be involved in the inhibition of tyrosine phosphorylation in pregnant *db*/+ mice and restoration in the *db*/+TG6 mice, in addition to increased GLUT4 levels.

One of the interesting findings of these studies is that overexpression of GLUT4 in *db*/+TG6 mice was associated with highly improved insulin secretion during the glucose tolerance test. Significant questions still remain regarding the mechanism whereby improved insulin sensitivity can reduce β-cell abnormalities in diabetes. The *db*/+TG6 mice had fasting glucose levels of 50 mg/dl, and glucose remained <200 mg/dl throughout the glucose tolerance test. The glucose-lowering effect in the *db*/+TG6 mice presumably is a partial result of improved muscle insulin resistance that protects the pancreas from hyperglycemia-dependent loss of glucose responsiveness (37), thereby preventing the mice from developing defects in insulin secretion. The GLUT4 transgenic mice used in these studies were carrying 11.5 kb of the human GLUT4 genomic DNA, including 5.4 kb of the endogenous GLUT4 promoter. There is good evidence that the pattern of tissue-specific expression of the 11.5-kb construct follows that of the endogenous mouse GLUT4 gene (32). Developmental studies have shown that GLUT4 gene expression in skeletal muscle is absent in fetuses at birth (44); it progressively increases during the suckling period. Because the increases in GLUT4 expression are primarily limited to the maternal transgenic mice at the time of these studies, the adaptations observed during pregnancy are primarily due to increased maternal GLUT4 expression. In previous studies using *db/db* mice, overexpression of GLUT4 markedly increased insulin levels and lowered glucose in homozygous, obese-diabetic *db/db* mice. *db/db* mice have a propensity for developing impaired insulin secretion as they age (25,26). Although GLUT4 has not been found in pancreatic tissues, *db/db*GLUT4 mice had a threefold increase in pancreatic islet mass compared with nontransgenic mice (30). These data suggest that overexpression of human GLUT4 results in a more functional pancreatic β-cell that can respond more efficiently to elevated glucose and other substrates coming from increased food intake in pregnant *db*/+ mice. Along these lines, it is known that lean GDM patients, in addition to retaining more insulin resistance, have inadequate insulin secretion after pregnancy (11,12). By extrapolating the present studies to human pregnancies, it can be inferred that susceptibility to GDM is due to a synergism between inadequate insulin secretion and impaired activation of the insulin-signaling pathway, leading to decreased glucose uptake in skeletal muscle. These studies also suggest that therapies aimed at increasing GLUT4, such as exercise training (45), may decrease the risk for GDM in patients predisposed to developing diabetes in pregnancy.

We observed that pregnancy increases circulating leptin concentration more than 40-fold in wild-type +/+ and *db*/+ mice. Gavrilova et al. (46) recently demonstrated that murine placenta, unlike human placenta, does not express the leptin gene. Rather, pregnancy in mice is associated with the appearance of large amounts of the circulating OB-Re (short form) of the leptin receptor which acts as a soluble binding protein in the serum during gestation. Gavrilova et al. (46) showed that leptin binding capacity in the serum rises ~40-fold by day 18 of gestation and suggested that these binding proteins contribute to reduced clearance of leptin. Our results demonstrate that hyperleptinemia of pregnancy is significantly greater during diabetic pregnancy and significantly reduced in *db*/+TG6 mice. These findings suggest that

diabetes may contribute to the regulation of the expression of these binding proteins. It is not known whether increased production of leptin binding proteins plays a role in maternal weight gain and transfer of maternal nutrients across the placenta. The fact that increased energy intake and weight gain continued throughout pregnancy suggests that maternal leptin resistance may be an important metabolic adaptation necessary for maintaining a viable pregnancy. To date, the identity of leptin binding proteins and their effect on leptin signaling remain undefined in the nonpregnant state, let alone during pregnancy. In most cases, secreted forms of other cytokine receptors tend to chelate the ligand and act as inhibitors. This could also be possible with leptin. Excess leptin-binding proteins during pregnancy might possibly contribute to decreased leptin action through downregulation of local leptin receptor signaling necessary for requisite adaptive changes that occur during pregnancy. However, the functional significance of leptin receptors and placental leptin during pregnancy remains to be determined.

Maternal hyperglycemia is a major factor associated with adverse perinatal outcome, particularly macrosomia (47,48). Significant correlation also exists between the levels of certain maternal amino acids, free fatty acids, and triglycerides and newborn weight (49,50). However, the relative contribution of each of these substrates on fetal birth weight has yet to be defined. We found that *db*/+ and *db*/+TG6 mice had the same litter sizes as wild-type +/+ mothers; however, both *db*/+ and *db*/+TG6 mothers delivered pups that were more than 2 SD greater than the mean birth weight of pups born to +/+ mothers. The results in *db*/+TG6 mothers suggest that macrosomia may occur despite normoglycemia. The reasons for increased fetal mass in pups born to *db*/+TG6 mice are not clear; however, one explanation for increased fetal mass may be that adding excess substrates to the maternal compartment may increase placenta growth and transfer of substrates to the fetus. During pregnancy, the *db*/+TG6 mice had a significantly lower weight gain and fat mass compared with +/+ mice, despite significantly greater food intake. Studies are currently underway in our laboratory to determine whether matching energy intake in *db*/+ mice to the levels in +/+ mice during pregnancy will decrease fetal overgrowth.

At the other extreme, we observed that *db*/+TG8 mice, which have four- to fivefold greater GLUT4 protein expression, gave birth to fewer pups that weighed significantly less than pups from control +/+ mothers. It is well known that limitations in substrate availability (mothers with hypoglycemia) during pregnancy are more likely to have small-for-gestational-age (SGA) neonates (47,51–53), and this is a significant source of perinatal morbidity and mortality. The mechanisms underlying SGA are not well understood. In the *db*/+TG8 mice, the overexpression of GLUT4 results in a fourfold increase in muscle plasma membrane GLUT4 (30). Our data in Table 2 showing lower insulin and glucose levels are consistent with previous observations showing that insulin-stimulated glucose disposal is significantly elevated in TG8 compared with nontransgenic mice, with an intermediate level in the TG6 mice consistent with the intermediate level of GLUT4 expression. As a result, fasting plasma glucose values in the nonpregnant state are ~40 mg/dl, insulin levels are substantially lower, and the glucose tolerance curve is nearly flat in *db*/+TG8 mice. These findings make it tempting to speculate that highly increased GLUT4 expression leads to a shunting of substrates into the maternal compartment away from the fetus. It was also noted that a transgenic line with eight- to ninefold increase in GLUT4 expression could not be propagated as GLUT4 homozygotes, as the transgenic dams died of unknown complications shortly after giving birth (E.M.G., S.C. McCoid, P.D. McGill, unpublished observations). These data suggest that increased GLUT4 expression, while increasing insulin-signaling, reducing the stress on the pancreatic β-cell, and lowering maternal glucose, at some point may pose an increase risk for maternal and fetal complications.

In summary, the present results suggest that the single mutated *db* allele, when propagated on the C57BLKS/J background, can confer a dominant effect on susceptibility to gestational diabetes associated with abnormalities in insulin receptor signaling, decreased insulin secretion, and greater nutrient availability. These results suggest that increased GLUT4 expression can improve insulin-signaling in diabetic mice, resulting in increased insulin secretion and markedly improved glycemic control during pregnancy. The results in *db*/+TG6 mothers suggest, however, that maternal hyperglycemia may not be the sole cause of fetal macrosomia.

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